Binding of Antitumor Antibiotic Daunomycin to Histones in Chromatin and in Solution†

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ABSTRACT: Daunomycin is an anticancer drug that is well-known to interact with DNA in chromatin. Using a compositionally defined chicken erythrocyte chromatin fraction, we have obtained conclusive evidence that the drug is also able to interact with chromatin-bound linker histones without any noticeable binding to core histones. The drug can interact in an equal fashion with both histone H1 and H5 and to a greater extent with core histones H3/H4 and H2A/H2B as free proteins in solution. Thus, the binding of daunomycin to linker histones in the chromatin fiber is most likely due to the well-known higher accessibility of these histones to the surrounding environment of the fiber. Binding of daunomycin to linker histones appears to primarily involve the trypsin-resistant (winged-helix) domain of these proteins. The studies described here reveal the occurrence of a previously undisclosed mechanism for the antitumor activity of anthracycline drugs at the chromatin level.

The anthracycline antibiotic daunomycin and its derivatives are antitumor drugs widely used in cancer chemotherapy to treat myelogenous leukaemia and solid tumors (*1*−*3*). There is plenty of evidence indicating that cellular DNA is the primary target for these drugs. Daunomycin acts by intercalation of its planar aglycan chromophore between DNA base pairs, and its amino sugar ring lies in the minor groove of the double helix (*4*−*9*). The binding of daunomycin to DNA results in the inhibition of both RNA and DNA synthesis (*10*−*12*).

In the cell nucleus, DNA does not exist as a naked structure but is associated with basic proteins called histones forming discrete complexes known by the name of nucleosomes (*13*−*15*), which are the fundamental subunits of chromatin. Hence, in the eukaryote cell, chromatin should be thought of as the main target of action of anthracycline drugs (*16*). How the occurrence of histones that bind to DNA affects the binding of daunomycin is an important question when trying to understand the mechanism(s) of action of these drugs at the chromatin level. To explore this question, some authors have focused on the binding of daunomycin to chromatin and nucleosomes (*17*−*20*) and shown that the binding affinity of daunomycin to chromatin increases upon removal of histones. We have also shown that preferential binding of daunomycin to the linker DNA regions induces an unfolding of the chromatin fiber that is followed by aggregation (*21*).

We have recently focused our attention on the interaction of daunomycin with histone H1 (linker histones). We have shown that, similarly, to what is observed for the interaction with DNA (*6*), the binding of daunomycin to histone H1 stabilizes the protein against thermal denaturation (*22*) and the H1−daunomycin complex exhibits a positive cooperativity (*23*).

Histones of the H1 family are a very lysine-rich protein fraction of chromatin with a very different structure and function from that of core histones. They bind to linker DNA between adjacent nucleosomes to facilitate the folding of the chromatin fiber (*24*), and hence, they are also referred to as linker histones. The primary structure of these proteins is characterized by an uneven distribution of charged amino acid residues along the polypeptide chain. Linker histones exhibit a tripartite structural organization in solution. The amino and carboxyl termini are about 20−30 amino acids long. The central part of the molecule (of about 80 amino acids) is the least basic part, contains the bulk of the hydrophobic amino acids (*24*, *25*), and is resistant to digestion by trypsin. This region is followed by a highly charged random coil C-terminus region of about 100 amino acids, which consists of a large number of lysine, alanine, and proline residues. (*24*, *25*). The trypsin-resistant core region has a "globular" organization with a discrete number of well-defined secondary structure domains arranged in a winged-helix motif (*26*−*28*).

In this paper, we have extended our previous research on the interaction of daunomycin to histone H1 and we provide evidence for the binding of daunomycin to the linker histones in chromatin and when free in solution. The binding of the free drug to the trypsin-resistant core of H1 and H5 in comparison to the whole protein has also been characterized.

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MATERIALS AND METHODS

Materials. Daunomycin hydrochloride was purchased from Sigma (St. Louis, MO). A stock solution of drug was prepared in sterile distilled water at a concentration of 5 mg/mL and stored at −20 °C until further use. Dilutions of the drug stock in the appropriate buffer were prepared immediately before use. The concentration of daunomycin was determined spectrophotometrically using an extinction coefficient of 11 500 M^{-1} cm^{-1} at 480 nm.

Micrococcal nuclease was obtained from Worthington (Freehold, NJ). CM-Sephadex C-25 was from Amersham Biosciences, Inc. (Piscataway, NJ), and Hydroxyapatite (HAP) was from Bio-Rad Laboratories (Hercules, CA). Trypsin, protein inhibitor cocktail (complete), and proteinase K were purchased from Roche (Laval, QC). Chicken blood was collected from chickens obtained from local suppliers. Immediately after collection, a 10% volume of 0.15 M NaCl, 15 mM sodium citrate, and 10 mM sodium phosphate (pH 7.2) buffer (containing 150 units/mL of heparin) was added (*29*) and the cell suspension was centrifuged at 1500*g* for 10 min at 4 °C. The loose cellular pellet was mixed with one volume of buffer (without heparin), brought to 40% glycerol, and kept frozen at −80 °C until use.

Interaction of Daunomycin with Histones in Chromatin. Nuclei were prepared from chicken erythrocytes as described elsewhere (*29*). The nuclear suspension at $A_{260} = 100$ was digested with 6 units of micrococcal nuclease/mL for 10 min at 37 °C. The solution was then brought to 10 mM (ethylenedinitrilo)tetraacetic acid (EDTA)[1] on ice and centrifuged at 12000*g* for 5 min at 4 °C. The pellet was resuspended in 0.25 mM EDTA (pH 7.5), and the nuclei were lysed by gentle stirring at 4 °C for 1 h. The lysate was then centrifuged at 12000*g* for 15 min, and the chromatin in the supernatant was designated as EDTA-soluble chromatin (SE).

The concentration of DNA in the chromatin fractions was determined spectrophotometrically, using an extinction coefficient of 20 cm^2 mg^{-1} at 260 nm. Daunomycin was added to SE chromatin (100 μg/mg DNA) and incubated at 22 °C for 45 min. The mixture was then dialyzed overnight against 20 mM potassium phosphate buffer (pH 6.8) using dialysis tubing [Spectra/Por 3, molecular weight cut off of 3500 (Spectrum Laboratories, Inc., Rancho Dominguez, CA)]. The sample was then loaded onto a 15 × 1.5 cm HAP (hydroxyapatite) column that had been pre-equilibrated with the same buffer. The histones of the chromatin sample were eluted using a salt gradient of 0−2 M NaCl. The flow rate was 5 mL/h, and 1 mL fractions were collected. Absorbance was monitored at 230 and 480 nm. Desired fractions were pooled as indicated in the figure captions, and the fluorescence emission intensity of the selected fractions was measured (see below). To mimic the physiological environment of chromatin, the SE fraction was dialyzed against 75 mM NaCl and 20 mM potassium phosphate buffer (pH 6.8) and treated with daunomycin under the same conditions as described above. After treatment, the sample was dialyzed again against 75 mM NaCl and 20 mM potassium phosphate buffer (pH 6.8) and histones were eluted from an HAP column equilibrated in this buffer and eluted with a 75 mM to 2.0 M NaCl gradient.

Interaction of Daunomycin with DNA. DNA from the SE chromatin fraction was isolated by the proteinase K digestion and phenol-chloroform extraction procedure described previously (*21*). Purified DNA was then dissolved in 20 mM potassium phosphate buffer at pH 6.8, and its concentration was determined spectrophotometrically. The DNA sample thus obtained was mixed with daunomycin, treated under the same conditions as the SE chromatin (see above), and loaded onto a HAP column that was also eluted in a similar fashion as described for SE chromatin.

Isolation of Histones H1 and H5 and Preparation of their Trypsin-Resistant Core. Nuclei was prepared from chicken blood and digested with micrococcal nuclease as described before (*29*). Histones H1 and H5 were extracted from the SE chromatin fraction using 0.35 M NaCl in the presence of 12 mg of CM-Sephadex C-25/mg of DNA, and the resin-bound histones were further purified using CM-Sephadex C-25 ion-exchange chromatography as described elsewhere (*30*). The purified proteins were then dialyzed against distilled water and freeze-dried.

The purified histones H1 and H5 were separately digested with trypsin in the presence of 2 M NaCl, essentially as described by ref *31*. The enzymatic digestion was carried out for 30 min at 22 °C, and the trypsin-resistant core of the proteins was then precipitated with 18% trichloroacetic acid and fractionated on CM Sephadex C-25 column under the conditions described by ref *32*.

Gel Electrophoresis. Sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE, 15% acrylamide and 0.8% bisacrylamide) was carried out as described by Laemmli (*33*). Acetic acid (5%) and urea (2.5 M) PAGE was performed as described elsewhere (*34*). The gels were stained with 0.1% Coomassie B Blue and destained with a 10% acetic acid and 10% 2-propanol solution.

Interaction of Daunomycin with Histones. The histones H1 and H5 and their respective trypsin-resistant cores (H1C and H5C) were each dissolved in 20 mM sodium phosphate buffer (pH 7.2), and their concentration was determined spectrophotometrically. The extinction coefficient used for H1 was 1350 cm^{-1} M^{-1} at 276 nm (*35*). An extinction coefficient of 12015 cm^{-1} M^{-1} at 230 nm was calculated for the protein using this value and the A_{230}/A_{276} ratio experimentally determined. For histone H5, we used an extinction coefficient of 1.85 cm^2 mg^{-1} at 230 nm (*36*), which corresponds to 38 100 cm^{-1} M^{-1}. The extinction coefficient of the H5C was calculated from the experimentally determined A_{230}/A_{276} (a), for the whole histone H5, and A_{230}/A_{276} (b), of its trypsin-resistant core determined under the same buffer conditions, $\epsilon_{230}(H5C) = \epsilon_{230}(H5)$. The value determined in this way was 28 250 cm^{-1} M^{-1}. Also, the extinction coefficient of H1C was calculated and was 11 277 cm^{-1} M^{-1} . Appropriate concentrations of daunomycin (5−150 μM) were incubated with each protein (5−11 μM) for 30−45 min at 22−25 °C in the dark. Free daunomycin and also histones (without the drug) were prepared in the same buffer and incubated along with the drug−histone samples under the same conditions and used as the controls.

Spectroscopy: UV Spectroscopy. Histones and daunomycin were mixed as described above in 20 mM sodium phosphate

[1] Abbreviations: CD, circular dichroism; EDTA, (ethylenedinitrilo)-tetraacetic acid; HAP, hydroxyapatite; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulphate; UV, ultraviolet.

buffer (pH 7.2), and the spectrophotometric measurements were carried out at 20−23 °C. The absorbance was measured at 210 and 480 nm using a Cary 1, Varian UV−vis spectrophotometer (Varian Inc, Palo Alto, CA). The changes in absorbance were calculated by subtracting the drug (or protein) absorbance at each point from that of the protein−drug complex, and the results were normalized for the protein concentration.

Fluorescence. The measurements were performed on a Photon Technology International Apparatus (West Sussex, U.K.) using quartz cells of 1 × 1 cm path length. The monochromator slits were set at 2−5 nm to reduce the intensity of the signal depending on the experiment. Experiments were performed at 20 °C using a thermostat, and all solutions were made in 20 mM sodium phosphate buffer (pH 7.2). The protein concentration of all of the samples analyzed was 7 μM, while the drug concentration ranged between 0 and 28 μM. After addition of the drug, the samples were allowed to equilibrate for 20 min before the fluorescence measurements were taken. Daunomycin and the histone solutions were prepared in the same buffer and used as a control. Tyrosine was also dissolved in the same buffer, and its emission spectrum was recorded in the same conditions and used as a reference. The spectra were recorded between 290 and 370 nm for the proteins after excitation at 278 nm. In the case of daunomycin, a wavelength range of 500−700 nm after excitation at 485 nm was used.

Circular Dichroism (CD). CD spectra were recorded on a JASCO J-720 spectrophotometer (Hachioji, Tokyo, Japan) as described elsewhere (*37*). The spectra were recorded from 190 to 260 nm using a 10 nm path-length cell. Various concentrations of daunomycin were added to a fixed histone concentration (30−40 μM) in 20 mM sodium phosphate (pH 7.2) buffer, and the spectra were recorded at a scan speed of 2 nm/min and constant temperature of 20 °C.

RESULTS

Daunomycin Preferentially Binds to Linker Histones in Chromatin. The binding of daunomycin to histone H1 in solution has already been partially characterized (*22*, *23*). However, to date, there is no evidence of binding of daunomycin to histones, including the members of the histone H1 family within the chromatin context.

To elucidate this possibility, an experiment was designed using an SE chicken erythrocyte chromatin fraction incubated in the presence of an excess amount of daunomycin. The excess of unbound drug was removed by extensive dialysis, and the histones from the chromatin−drug complex were fractionated by HAP chromatography. Elution was carried out using a salt gradient, and the amount of drug bound to the fractions collected was analyzed by fluorescence spectroscopy. Figure 1A shows the elution profile thus obtained, which consists of three main peaks: A, B, and C. It should be noted that under the experimental conditions used, DNA remains bound to the resin and histones come off the column, as the salt concentration increases. Selected fractions from the elution profile were pooled, and their fluorescence emission intensity was measured. For these determinations, the excitation was at 485 nm and fluorescence emission was measured between 500 and 700 nm. This is the specific wavelength for daunomycin, and proteins did not exhibit any fluorescence within this range. The fluorescence intensities were normalized to the protein concentration in each fraction. As seen in Figure 1B, there is a considerable difference between the different fractions. No fluorescence up to fraction 68 could be detected. The highest fluorescence observed corresponded to peak A, while peaks B and C showed only very low intensity. The rest of the fractions throughout the chromatogram showed very little or no intensity.

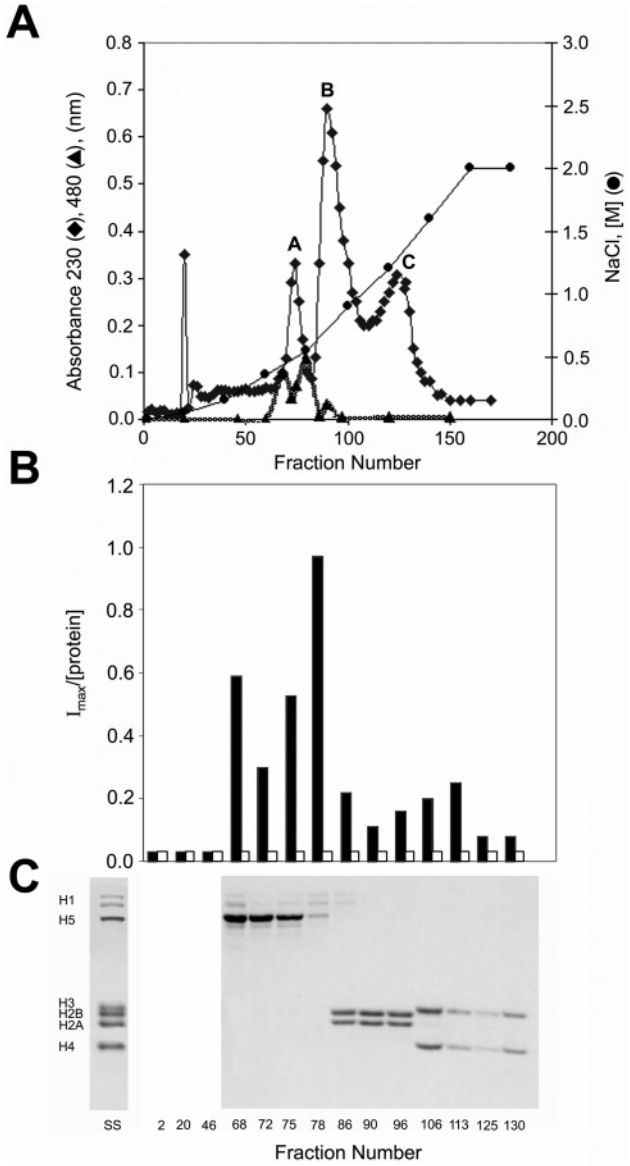

FIGURE 1: Fractionation of daunomycin-treated chicken erythrocyte chromatin. (A) Hydroxyapatite chromatography. A linear salt gradient of 0−2 M NaCl in 20 mM K phosphate buffer (pH 6.7) was used for the elution of the histones. Fractions were pooled for fluorescence analysis and designated as **2** = *1−4*, **20** = *20−24*, **46** = *46−49*, **68** = *67−70*, **72** = *71−74*, **75** = *75−77*, **78** = *78−80*, **86** = *85−87*, **90** = *88−93*, **96** = *94−99*, **106** = *105−109*, **113** = *112−116*, **125** = *124−128*, and **130** = *129−133*, where the bold numbers correspond to the fraction numbers shown in B and C and the italic numbers are the pooled fractions from the HAP in A. (B) Fluorescence emission intensity (I_{max}) of selected fractions normalized with respect to the corresponding protein concentration. Excitation was at 485 nm. The black bars correspond to SE chromatin fractions, and the white bars are the fractions obtained from a daunomycin-treated DNA eluted and treated under the same conditions as the SE chromatin. (C) SDS−PAGE of the fractions used in the fluorescence analysis. SS is the starting sample before HAP fractionation.

Figure 1C shows the SDS gel electrophoresis pattern of the fractions used for this analysis. As seen there, fractions 68−79 consist of histones H1 and H5; however, these histones are not evenly distributed. While fractions 68 and 75 contain both histones, fraction 72 consists of a very low abundance (but not complete absence) of H1 relative to H5 and fraction 78 is highly enriched in histone H1 compared to the starting sample. It is also important to notice that fraction 86 that consists mainly of histone H2A and H2B but contains some histone H1 exhibits a higher fluorescence than fraction 90 consisting only of histones H2A and H2B. Interestingly, the fractions with a higher amount of H1 are those that exhibit higher fluorescence. From this analysis, it seems as if there are two different kinds of histone H1, which elute at different salt concentrations. In contrast, fractions 86, 90, and 96 corresponding to histones H2A and H2B and fractions 106−130 consisting of histones H3 and H4 exhibit very little intensity compared to histones H1 and H5. The fluorescence results for the binding of daunomycin to histones are in good agreement with the absorbance profile at 480 nm of the corresponding fractions (Figure 1A). Very similar results to those described above were obtained when the ionic strength of the SE chromatin sample was adjusted to physiological values (see the Materials and Methods, results not shown) prior to loading onto the HAP column. This precludes the existence of nonspecific binding effects resulting from the binding of the drug to chromatin under the low ionic strength conditions of the SE chromatin sample.

To ensure that the fluorescence intensity observed is not due to the gradual release of the drug from chromatin DNA as a result of the salt gradient used, DNA was also isolated from the EDTA-soluble (SE) chromatin fraction (the same chromatin fraction used above). This DNA sample was treated with daunomycin and chromatographically fractionated under the same conditions as SE. The fluorescence emission intensity of the fractions was measured, and the results are also shown in Figure 1B. No fluorescence intensity was observed in any of the fractions collected from this elution.

Solution Studies of the Interaction of Daunomycin with Histones H1 and H5 and Their Respective Trypsin-Resistant Cores. To further characterize the binding of daunomycin to the histones of the H1 family and in an attempt to elucidate the molecular mechanisms involved, histones H1 and H5 as well as their respective trypsin-resistant cores were prepared as described in the Materials and Methods. The purity of the proteins was assessed using SDS and acid−urea gel electrophoresis (see parts A and B of Figure 2). As seen in Figure 2B, the trypsin-resistant cores (H1C and H5C) were quite homogeneous and exhibited the same electrophoretic mobility as that previously described for these peptides (*30*, *32*).

Spectroscopy [absorbance in the ultraviolet (UV) region] was successfully used in the past for the analysis of daunomycin−DNA (*5*) and daunomycin−histone (*23*) interactions. To this end, the purified proteins (with an absorbance of 1.5 at 210 nm) were incubated in the presence of various concentrations of daunomycin in the dark, and the changes in their UV absorbance at 210 nm were measured. The absorbance changes were calculated by subtracting the absorbance of the free drug from the absorbance of the protein−drug complex and normalized

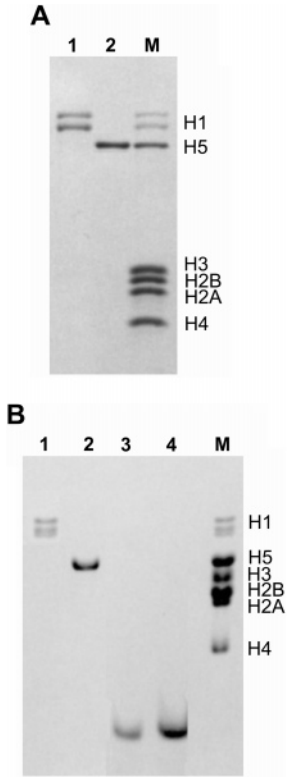

FIGURE 2: Electrophoretic analysis of the histones used for the characterization of the protein−daunomycin interaction in solution. (A) SDS−PAGE of the purified histones H1 (lane 1) and H5 (lane 2). (B) Acetic acid−urea PAGE of histones H1 (lane 1) and H5 (lane 2) and their respective trypsin-resistant cores, H1C (lane 3) and H5C (lane 4). M is a chicken erythrocyte histone marker.

with respect to the protein concentration. The results thus obtained are shown in Figure 3. Figure 3A shows the absorbance changes of histones H1, H5, and H5C as a function of the drug concentration. As seen in this figure, the absorbance pattern exhibited by all of these proteins was very similar. When the absorbance of the protein was subtracted from the absorbance of the protein−drug complex, only a slight decrease in the drug absorbance at 210 nm was observed (Figure 3C).

Fluorescence spectroscopy has emerged as one of the basic techniques to probe and provide information on the structural properties of biological macromolecules. To further study the binding of daunomycin to histones, various amounts of daunomycin (3−50 μM) were added to a constant concentration of these proteins (7 μM) and the fluorescence emission was determined. The excitation of the proteins was carried out at 278 nm, and the fluorescence was recorded in the range of 290−370 nm, which is entirely contributed by the tyrosine residues because the phenylalanine contribution is considered negligible and tryptophan is absent from these proteins. The results thus obtained, are summarized in Figure 4. Figure 4A shows the fluorescence emission spectra of histone H1 in the presence and absence of various concentrations of daunomycin. The fluorescence spectrum of tyrosine has also been provided for comparison. As expected, histone H1 exhibits an emission spectrum in the position corresponding to tyrosine with a maximum intensity at 305 nm. Addition of daunomycin to the H1 solution (drug−protein molar ratios of 1, 2, and 4) decreased the fluorescence emission intensity of histone H1 as the drug concentration was increased. The

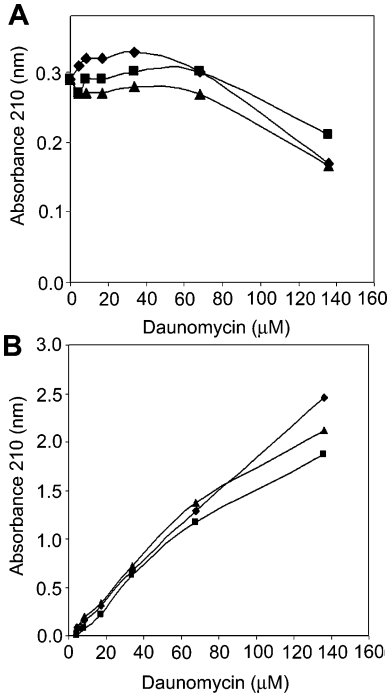

FIGURE 3: UV absorbance characterization of the protein−daunomycin interaction in solution. (A) Absorbance at 210 nm after subtraction of the drug contribution and normalization with respect to the protein concentration. (B) Absorbance at 210 nm upon subtraction of the protein absorbance contribution. The UV absorption studies were carried out in 20 mM sodium phosphate (pH 7.2) buffer. Histone H1 (◆), histone H5 (■), and H5C (▲).

fluorescence emission spectra of histone H1 and its trypsin-resistant core (H1C) are shown in Figure 4B. For simplicity, only the spectra at the 1:4 (histone/daunomycin) molar ratio are shown. Histone H5 and H5C (Figure 4C) exhibited nearly the same fluorescence emission spectra, and as with histone H1, the maximum intensity occurred at 305 nm and addition of the drug reduced the fluorescence intensity in a similar way. The similarity of the fluorescence quenching by the drug in the intact histones and their trypsin-resistant cores can be better appreciated in Figure 4D.

As it has already been pointed out earlier in the analysis of the interaction of daunomycin with histones in chromatin (see Figure 1), histones H2A/H2B and H3/H4 exhibited a very low but noticeable fluorescence intensity. Therefore, we decided to analyze also the interaction of daunomycin with these histones in solution. The results are summarized in Figure 4D. Although the fluorescence intensity of H2A/H2B and H3/H4 are the same, the reduction in fluorescence intensity was higher in these core histones compared to the linker histones.

To determine if the binding of daunomycin to linker histones has any effect on their secondary structure, we determined the CD spectra of the histone H1 and H1C in the presence and absence of daunomycin. Various concentrations of daunomycin were added to a constant concentration of each protein (ca. 30−40 μM), and the spectra were recorded between 200 and 260 nm. The results are shown in parts A and B of Figure 5. The spectra of these proteins in the absence of any drug were very similar to those reported previously (*35*, *38*). Upon addition of daunomycin, the ellipticity at 208 nm changed significantly, while the overall ellipticity at 220 nm remained virtually unchanged.

DISCUSSION

The binding of daunomycin to chromatin has been the subject of many reports, which have consistently shown that the drug has less affinity for the nucleosomes than for naked DNA (*10*, *18*, *21*). Despite these studies, to date, there is no direct evidence of binding of the drug to histones in chromatin. In this work, the binding of daunomycin to histones in a soluble chromatin fraction and in solution has been investigated.

The histone HAP fractionation of daunomycin-treated chromatin (Figure 1) conclusively shows that, besides DNA, daunomycin also binds to the histone component of chromatin. This binding is predominantly targeted to linker histones with a clearly enhanced preference for histone H1 over histone H5.

The elution of the linker histones as a single peak (peak A in Figure 1A) is in contrast to what happens to the same native chromatin fraction in the absence of daunomycin, where linker histones elute as a double peak in this region, with the first peak corresponding to histone H1 and the second corresponding to histone H5 (results not shown). The differential elution of histone H1 at 0.45 and 0.58 M NaCl observed here obviously reflects differences in the interaction of this histone with daunomycin within the chromatin context, and it is likely the result of different mechanisms of binding. Our previous solution studies have shown the occurrence of two binding sites for daunomycin in histone H1 with two different association constants (*23*). Therefore, it is possible that the early and late elution fractions correspond to histones consisting of daunomycin occupying one or both of these sites (i.e., histones with a different extent of drug binding).

It is important to note here that the stoichiometry of linker histones (H1 and H5) to nucleosome in chicken erythrocytes is approximately 1.3 (0.9 mol of H5/0.44 mol of H1) (*39*). It has also been suggested that two binding sites with different affinity exist in the nucleosome for the binding of linker histones (*40*). Hence, it is likely that a fraction of the chicken erythrocyte linker histones (probably H1) occupies a secondary position in the nucleosome. The difference in the nucleosome location may account for the different accessibility of histone H1 to the drug within the chromatin context. Alternatively, it might be that the differential drug binding to H1 results in altered binding affinity for the nucleosome. As noted below, our results to date do not distinguish between these two possibilities.

The solution studies carried out with purified histone fractions show that the binding of daunomycin to native histones H1 and H5 and their corresponding trypsin-resistant cores are very similar, as indicated by both the absorbance (Figure 3) and fluorescence (Figure 4) data. The UV absorption results for histone H1 presented here are very similar to those previously reported (*23*).

The fluorescence emission spectra of all proteins studied here exhibit (in the absence of daunomycin) a characteristic fluorescence emission intensity maximum at 305 nm corresponding to the maximum fluorescence emission of tyrosine (*41*, *42*). Chicken erythrocyte histone H1 contains only one tyrosine and one phenylalanine. In contrast, histone H5 contains three tyrosines and one phenylalanine (*43*). In both instances, these aromatic amino acids are located in the

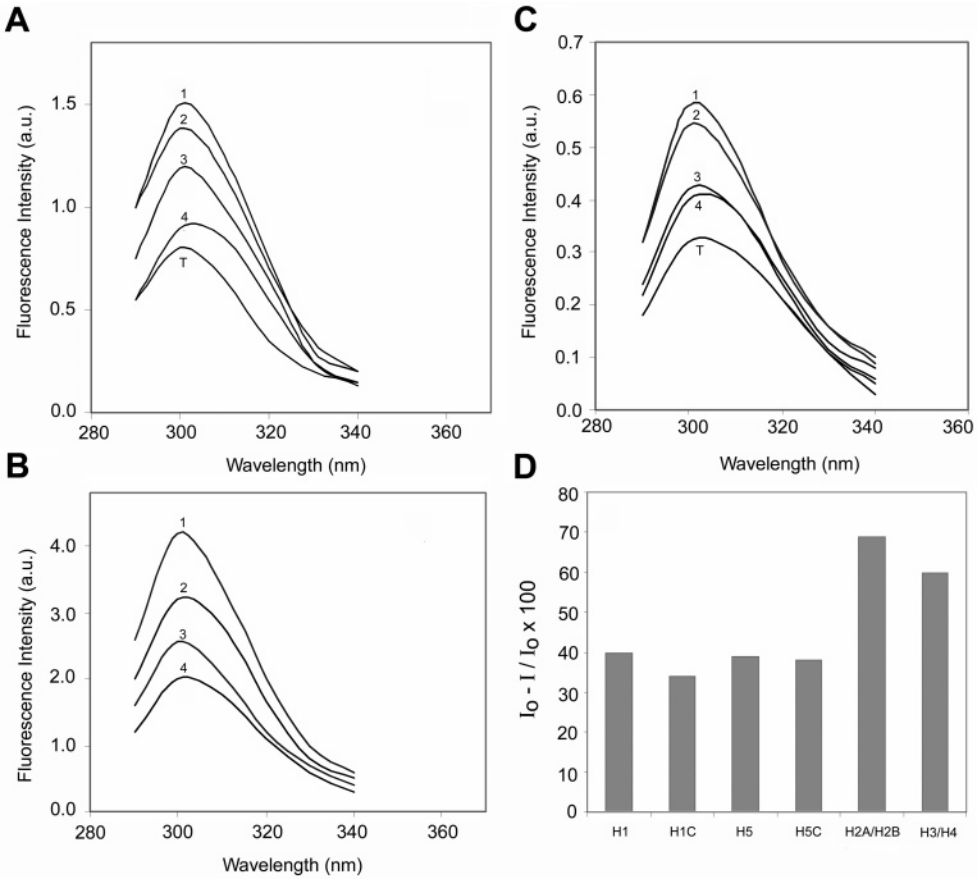

FIGURE 4: Fluorescence characterization of the histone−daunomycin interaction in solution. (A) Fluorescence emission spectra of histone H1 at (1), 0; (2), 7; (3), 14; and (4), 28 μM daunomycin. T is the spectrum of tyrosine. (B) Comparison of the fluorescence emission spectra of intact histone H1 (1 and 3) with its trypsin-resistant core H1C (2 and 4) in the absence (1 and 2) and presence (3 and 4) of 14 μM of daunomycin, respectively. (C) Fluorescence emission spectra of histone H5 and H5C in the absence (1 and 2) and presence (3 and 4) of 14 μM daunomycin, respectively, under the same conditions as in A. (D) $(I_0 - I)/I_0$ values for different histone fractions normalized with respect to the fluorescence of the protein in the absence of the drug. I_0 = fluorescence intensity before the addition of daunomycin, and I = florescence intensity after the addition of daunomycin. In all of the spectra shown, the excitation was at 278 nm and the samples were analyzed in 20 mM sodium phosphate (pH 7.2) buffer. The abbreviation (a.u.) is for arbitrary units.

trypsin-resistant core. However, whereas tyrosine is exposed to the solvent, in histone H1 (*44*), the three tyrosine residues of H5 are buried in the core (*45*). Addition of increasing amounts of daunomycin to these histones in solution at a constant protein concentration resulted in a reduction of their emission intensity without any red shift in the emission maxima (see parts A−C of Figure 4). A similar behavior was observed for H1, H1C, H5, and H5C, except for the fact that the emission intensities of H5 and H5C were much lower than that of H1 and H1C as expected from the 3D arrangement of their tyrosines. Indeed, when the spectra of the H1 and H5 were determined in the presence of 8 M urea, the emission spectra of H5 increased by 2−3-fold compared to the H1 (data not shown). When the fluorescence results were normalized and $(I_0 - I)/I_0$ were calculated for all of these proteins, all of them exhibited a very similar emission reduction (see Figure 4D).

Analysis of the fluorescence data for histone H1 using the procedure described by Ward (*46*) provided evidence for the existence of two binding sites with high and low binding constants (results not shown), in agreement with our previously published results using equilibrium dialysis (*23*).

Similar experiments were carried out with H3−H4 and H2A−H2B core histone fractions (Figure 1C). The reduction in fluorescence emission resulting from the quenching of the tyrosine residues of these molecules was about twice that exhibited by linker histones (Figure 4D). Despite their ability to interact with daunomycin in solution, neither H2A−H2B nor H3−H4 appear to be accessible to the drug in a nucleosome environment. When all of these results are taken together within the chromatin context, it appears that daunomycin binds to histones in a way that is dependent on their accessibility to the medium. The stronger interaction with histone H1 (Figure 1) and the occurrence of two distinct binding affinities (fractions 68 and 78 in Figure 1) suggest a more accessible location of H1 in chicken erythrocyte chromatin, which probably involves two different locations. Alternatively and/or complementarily, binding of daunomycin to the most accessible H1 may result in a tighter binding to chromatin, resulting in a late salt elution from the HAP column.

We also tried to determine if daunomycin binding results in any changes in conformation of the linker histones upon binding. The interpretation of the CD results (see Figure 5) is not straightforward. However, in contrast to other studies performed for the interaction of model tricyclic compounds with bovine serum albumin (*47*), very few changes occur in the α-helical structure of the molecule (as visualized by the lack of change in the ellipticity at 220−222 nm) (*37*) below a stoichiometry of approximately 1 mol of drug/mol of H1

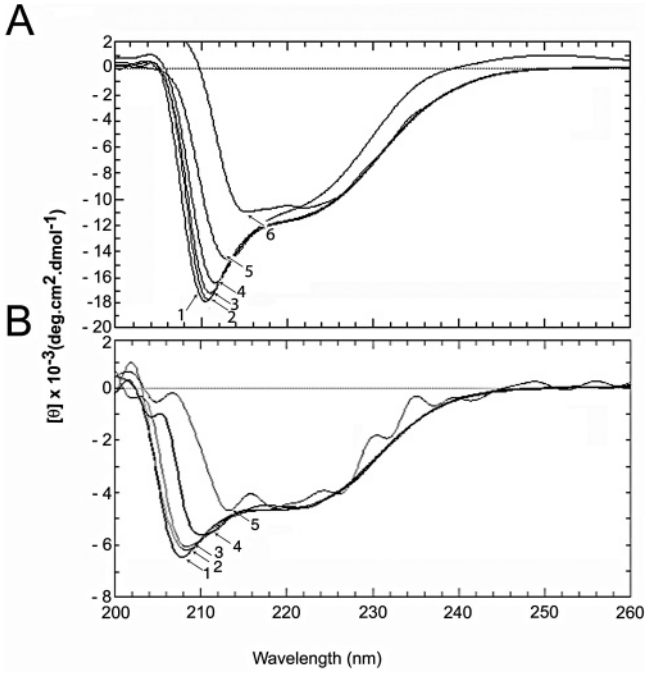

FIGURE 5: CD spectra of histone H1 and its trypsin-resistant core H1C in the presence of different concentrations of daunomycin. (A) Spectra of histone H1 (41 μM) at (1), 0; (2), 5; (3), 10; (4), 25; (5), 50; and (6), 100 μM daunomycin. (B) Spectra of histone H1C (33.6 μM) at (1), 0; (2), 10; (3), 25; (4), 50; and (5), 100 μM daunomycin.

(see parts A and B of Figure 5), suggesting that binding of the drug has little effect on the secondary structure of linker histones. Mock experiments carried out with histone H1 and another amine hydrochloride (i.e., triethanolamine hydrochloride) indicated that the changes observed in the spectra below 215 nm are most likely an artifact resulting from the presence of the amine hydrochloride. Sedimentation velocity experiments (results not shown) also ruled out the possibility that these changes are due to protein association/aggregation resulting from the interactions with the drug. Rather, a small trend in the increase of the sedimentation coefficient with the increase in the drug was observed, which suggests a process of compaction in the tertiary structure organization of the histones upon interaction with daunomycin.

It is finally worth pointing out that the interaction of daunomycin with linker histones in solution and as part of chromatin may have both a hydrophobic and partial electrostatic component because daunomycin has a positive charge. It is possible that the electrostatic component may facilitate the interaction of the drug with its primary binding site through interactions with the glutamic and/or aspartic acid in the vicinity of tyrosine within the trypsin-resistant core. The results shown in Figure 4D, strongly suggest that the trypsin-resistant core of these histones is the primary site of interaction. Interactions with acidic amino acids in the N- and C-terminal regions could also possibly occur and account for the two binding sites of this drug. Such interactions could contribute to the structural compaction observed in the analytical ultracentrifuge.

Within chromatin, daunomycin preferentially binds to the more accessible linker DNA domains, where it intercalates between the bases and changes the supercoiling of the fiber (*21*). Thus, it should not be surprising that it equally targets the histones bound to linker DNA (linker histones). The binding of anthracycline drugs to these histones adds yet another layer of complexity to the mechanisms involved in the still poorly understood antitumor action of these drugs at the chromatin level.

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